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Smooth Muscle Endothelin B Receptors Regulate Blood Pressure but Not Vascular Function or Neointimal Remodeling

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Abstract—The role of smooth muscle endothelin_B (ET_B) receptors in regulating vascular function, blood pressure (BP), and neointimal remodeling has not been established. Selective knockout mice were generated to address the hypothesis that loss of smooth muscle ET_B receptors would reduce BP, alter vascular contractility, and inhibit neointimal remodeling. ET_B receptors were selectively deleted from smooth muscle by crossing floxed ET_B mice with those expressing cre-recombinase controlled by the transgelin promoter. Functional consequences of ET_B deletion were assessed using myography. BP was measured by telemetry, and neointimal lesion formation induced by femoral artery injury. Lesion size and composition (day 28) were analyzed using optical projection tomography, histology, and immunohistochemistry. Selective deletion of ET_B was confirmed by genotyping, autoradiography, polymerase chain reaction, and immunohistochemistry. ET_B-mediated contraction was reduced in trachea, but abolished from mesenteric veins, of knockout mice. Induction of ET_B-mediated contraction in mesenteric arteries was also abolished in these mice. Femoral artery function was unaltered, and baseline BP modestly elevated in smooth muscle ET_B knockout compared with controls ($+4.2 \pm 0.2$ mmHg; $P < 0.0001$), but salt-induced and ET_B blockade-mediated hypertension were unaltered. Circulating endothelin-1 was not altered in knockout mice. ET_B-mediated contraction was not induced in femoral arteries by incubation in culture medium or lesion formation, and lesion size was not altered in smooth muscle ET_B knockout mice. In the absence of other pathology, ET_B receptors in vascular smooth muscle make a small but significant contribution to ET_B-dependent regulation of BP. These ET_B receptors have no effect on vascular contraction or neointimal remodeling. (*Hypertension*. 2017;69:275-285. DOI: 10.1161/HYPERTENSIONAHA.115.07031.) • [Online Data Supplement](#)

Key Words: autoradiography ■ endothelin-1 ■ hypertension ■ neointima ■ vasoconstriction

Endothelin-1 (ET-1), released by vascular endothelial cell (EC) and inner medullary collecting duct cells (and other cells under pathological conditions), stimulates endothelin_A (ET_A) and endothelin_B (ET_B) receptor subtypes.^{1,2} ET_A are present on vascular smooth muscle cells (VSMCs), predominantly mediating contraction³ and regulating blood pressure (BP).⁴ They also influence mitogenesis,⁵ generation of reactive oxygen species, and adhesion molecule expression.^{6,7} ET_A receptors on leucocytes mediate cytokine release and cellular chemotaxis.⁸ Many of these processes contribute to vascular

remodeling, and ET-1 clearly drives arterial lesion formation (including neointimal proliferation after injury).⁷ This can be inhibited by selective ET_A antagonism.^{9,10}

Regulation of arterial function, BP, and arterial lesion formation by ET_B receptors is likely to be more complex because they are expressed in EC, VSMC, and the kidney where they mediate physiologically antagonistic responses. ECET_B receptors mediate production of vasodilator, antiproliferative, and anti-inflammatory molecules (eg, nitric oxide [NO])^{11,12}; clearance of ET-1 from the circulation^{13,14}; and regrowth of damaged

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EC.¹⁵ VSMC ET_B can mediate vascular contraction, similar to the ET_A subtype,¹⁶ and may compensate for ET_A receptor dysfunction.¹⁷ ET_B upregulation in VSMC may mediate vasoconstriction and proliferation in cardiovascular disease.^{18,19}

ET_B-dependent regulation of BP is demonstrated by the sustained hypertension caused by ET_B receptor antagonism in mice.²⁰ The importance of receptor distribution in this response is indicated by increased BP after deletion of ET_B receptors in the renal collecting duct²¹ but not after deletion of ECET_B.²² The influence of VSMC ET_B on BP has not been established but, given their potential to mediate vasoconstriction, deletion or antagonism of VSMC ET_B would be predicted to reduce BP.

Despite the influence of ET-1 in vascular remodeling,²³ the role of ET_B is less clear. ET_B activation in EC (NO release) and kidney (reduced BP) would be predicted to inhibit arterial remodeling, thus favoring selective ET_A antagonism for reducing neointimal proliferation.⁹ Certainly, global deletion of ET_B receptors increases vascular lesion size.^{10,24} However, selective ECET_B deletion did not influence lesion formation, suggesting that the protective role was mediated by ET_B receptors in other tissues.⁹ If ET_B receptors in VSMC contribute to lesion formation, mixed ET_{A/B} antagonists might have advantages over ET_A selective compounds, although recent investigations^{9,10,24} favor the latter.

We generated novel smooth muscle ET_B receptor knockout (SMET_B KO) mice to address the hypothesis that loss of these receptors would impair arterial contraction, lower BP, and reduce neointimal lesion formation in response to vascular injury.

Methods

Mice with VSMC-selective ET_B receptor deletion were generated by crossing homozygous floxed ET_B mice with SM22cre transgenic mice, which express cre-recombinase in the heart and smooth muscle, (then backcrossed to a C57Bl/6J background for 4–6 generations), as described for ECET_B KO.²² Controls were Cre-negative littermates (ET_B^{fl/fl}). Genotyping was performed using ear clips.^{22,25} Wild-type (WT) C57Bl/6J mice were from Charles River (United Kingdom). Mice were housed according to the UK Home Office recommendations (22°C; 12-hour light/dark cycles) with free access to water and chow. Procedures were performed under the provisions of the Animals Scientific Procedures Act (1986) and approved by the local Ethics Committee.

Selective SMET_B deletion was demonstrated in organs and in isolated aortic smooth muscle cells (SMCs) using polymerase chain reaction, autoradiography,^{14,26} immunohistochemistry,²⁷ and functional (myographic) investigation of isolated trachea, arteries, and veins.^{28,29}

The impact of SMET_B KO on BP was assessed using radiotelemetry²² in conscious, unrestrained male SMET_B KO mice and age-matched controls (n=8 per group), fed on chow (7 days), high (7.6%) salt diet (7 days), then high salt plus ET_B antagonist (SB192621; 30 mg⁻¹ kg⁻¹ day⁻¹ in drinking water, 7 days). ET-1 concentrations in plasma from WT C57Bl/6J, controls, and SMET_B KO were measured after exposure to chow or to high salt diet plus ET_B antagonist, by ELISA (Endothelin-1 Quantikine ELISA kit; R&D Systems, Oxford, United Kingdom).

Intraluminal (left) or nondenuding (right) femoral artery injury was achieved by insertion of an angioplasty guidewire or ligation, respectively, as described.⁹ After 28 days, arteries were retrieved (after perfusion fixation) and analyzed using optical projection tomography, histology, and immunohistochemistry.^{9,30}

Statistics

Results are mean±SEM, for n mice. Group sizes were chosen to detect 5%, 20%, and 20% differences in BP (n=7), lesion size (n=7), and

maximum responses to vasoactive agents (n=6) with >90% power. Investigations were performed by operators blinded to treatment. Components of lesions were expressed as a percentage of the neointimal area. Analyses were performed with GraphPad Prism using Student *t* test, 1-way or 2-way ANOVA with a Tukey post hoc test, as indicated. Significance was assumed for *P*<0.05.

Detailed methods are in the [online-only Data Supplement](#).

Results

Identification of SMET_B KO

Genotyping for SM22cre, WT, and delta band alleles (Figure 1A) identified SMET_B KO (positive for SM22cre, floxed, and delta band and negative for WT allele) and controls (SMET_B^{fl/fl} cre-negative littermates; negative for WT allele, positive for floxed allele, and negative for SM22cre and delta band). SMC isolated from the aorta of SMET_B KO mice expressed the cre, delta, and flox bands, whereas controls did not express the cre and the delta bands (Figure 1B).

Autoradiography (Figure 1C) identified ET_B receptors in the gut lining, lung, and kidney. This signal was not diminished after SMET_B deletion. ET_B expression (real-time polymerase chain reaction) was not altered in the colon, heart, or gastrocnemius muscle of SMET_B KO mice (Figure S1 in the [online-only Data Supplement](#)). Confocal imaging of immunofluorescence (Figure 1D) clearly showed ET_B receptors localizing to the endothelium (von Willebrand factor positive) in SMET_B KO coronary artery. ET_B staining in medial SM remained at background levels. This confirms maintained ET_B receptor expression in the endothelium of SMET_B KO mice.

Functional Confirmation of SMET_B KO

SMET_B KO mice were healthy with normal body and organ weights (Table S1).

Sarafotoxin S6c (S6c)-mediated contraction in tracheas (which express ET_B receptors on SM)²² from controls was abolished by incubation with the selective ET_B antagonist A192621 (Figure 2A).²² In SMET_B KO mice, S6c-mediated contraction was reduced (≈30%), but not abolished. The residual contraction was blocked by ET_B antagonism. S6c-mediated contraction of mesenteric veins was abolished by selective deletion of SMET_B (Figure 2B).

SMET_B KO and BP

Control and SMET_B KO mice demonstrated a clear diurnal rhythm in BP (Figure 3A). Mean 24-hour BP was higher in SMET_B KO mice than in controls (107.1±0.3 versus 102.8±0.5 mmHg; n=7; *P*<0.0001; Figure 3B). Systolic BP was not different between groups (123.5±0.6 versus 124.8±0.5 mmHg; *P*=0.09; Figure 3C), but SMET_B KO mice had an increased diastolic BP (98.2±0.3 versus 92.2±0.4 mmHg; *P*<0.0001; Figure 3D). BP elevation occurred despite reduced heart rate (515±3 versus 538±5 bpm; *P*=0.004; Figure 3E). High salt increased BP in controls with a further increase induced by ET_B antagonism (Figure 4A). These responses were similar in SMET_B KO.

SMET_B KO and Circulating ET-1

Plasma ET-1 concentrations were similar in SMET_B KO and control mice (Figure 4B) and consistent with levels in WT C57Bl/6J (1.14±0.08; n=6). The combination of high salt diet

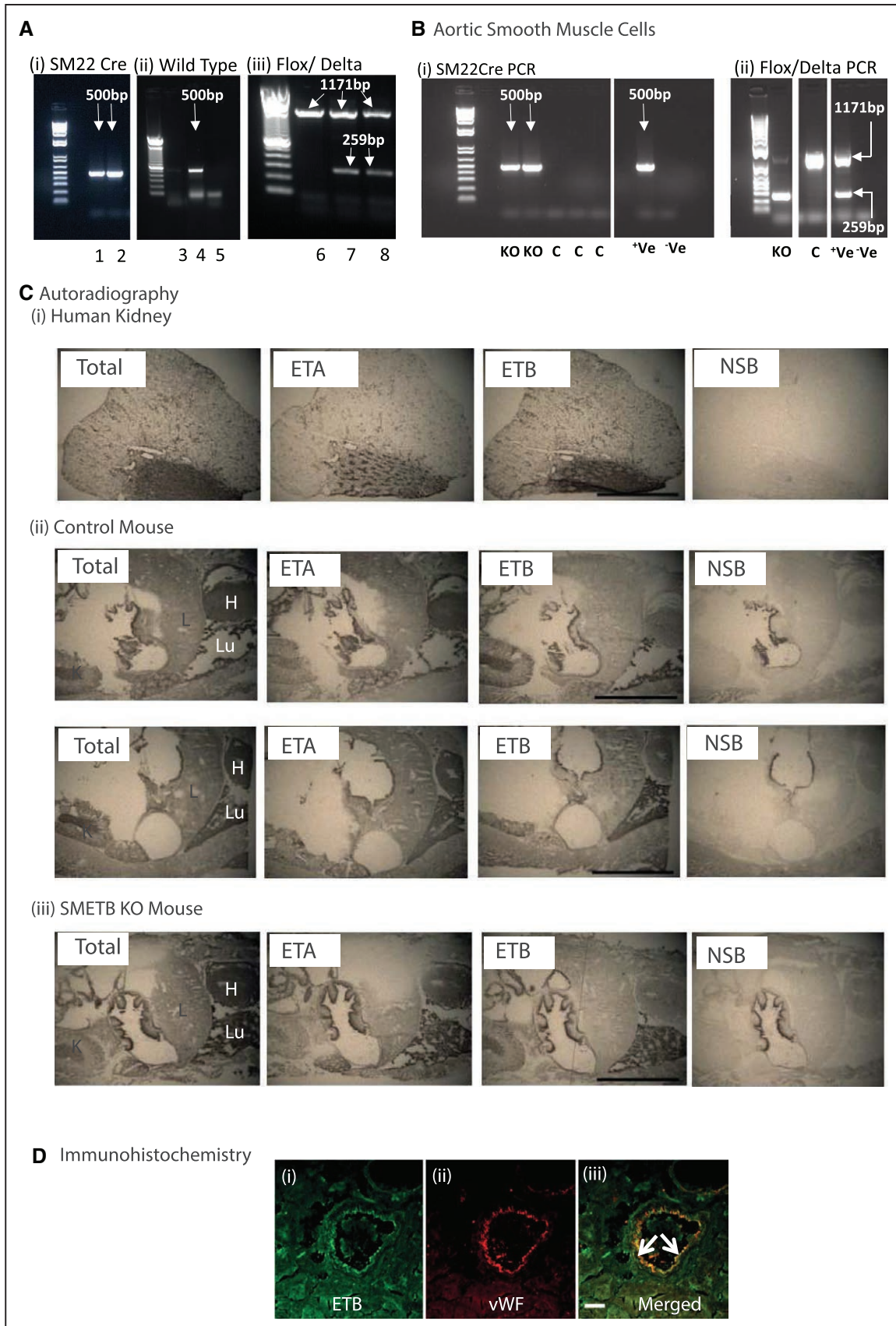


Figure 1. Selective endothelin_B (ET_B) receptor deletion from smooth muscle. **A**, Mice were genotyped for (i) SM22cre (band at 500 bp), (ii) wild-type (band a 500 bp), and (iii) flox (band at 1171 bp)/delta (band at 259 bp) alleles in ear clip DNA. (i) Samples 1 and 2 are cre-positive, (ii) sample 4 is positive for the wild-type allele; samples 3 and 5 are not, (iii) samples 7 and 8 are positive for both the flox (*Continued*)

Figure 1 Continued. and the delta band; sample 6 has only the flox band. **B**, Polymerase chain reaction (PCR) for cre and flox/delta bands in murine aortic smooth muscle cells isolated from smooth muscle ET_B receptor knockout (SMET_B KO) and control (C) mice. Control mice lacked cre and delta alleles, whereas SMET_B KO expressed all 3. Standard DNA ladders have band sizes 1500–100 bp. **C**, Autoradiography showing maintained ET_B ligand binding in SMET_B KO lung and kidney (representative of n=3 mice/genotype). **D**, Confocal images of a coronary artery from an SMET_B KO mouse stained for (i) ET_B receptor (green) or (ii) the endothelial cell marker von Willebrand factor (vWF; red). Merged images (iii) show clear colocalization of ET_B with the endothelium (arrows). There is no ET_B staining in medial smooth muscle. Scale bar=50 μ m. +Ve, positive control; –Ve, negative control; ET_A indicates endothelin_A; H, heart; K, kidney; L, liver; Lu, lung; and NSB, nonspecific binding.

and ET_B antagonism increased plasma ET-1 to a similar extent in control-type and SMET_B KO mice (Figure 4C).

SMET_B KO and Neointimal Remodeling

Wire injury of the left femoral artery generated neointimal lesions (Figure 5A).⁹ Optical projection tomography demonstrated that SMET_B KO altered neither the lesion volume (Figure 5B) nor cross-sectional narrowing (Figure 5C). Histological analysis showed a trend toward reduced cross-sectional narrowing in SMET_B KO (Figure 5D). Ligation of the right femoral artery generated lesions⁹ with similar volume

(Figure 5E) and maximal cross-sectional area (Figure 5F) in SMET_B KO mice and control mice.

Immunohistochemistry (Figure S2) showed that SMET_B KO did not differ from controls in the amount of macrophage (Mac-2; SMET_B KO 2.7 \pm 0.9% versus Control 2.6 \pm 0.7% lesion area), α -smooth muscle actin (SMET_B KO 14.8 \pm 4.1% versus Control 19.9 \pm 3.8% lesion area), or collagen (SMET_B KO 9.7 \pm 3.1% versus Control 14.9 \pm 3.2% lesion area) staining in the neointimal lesions.

SMET_B KO and Vascular Reactivity

In WT C57Bl/6J mice, EC removal from aortic rings abolished acetylcholine-mediated relaxation and enhanced the contractile response to phenylephrine but not to ET-1. EC removal from femoral arteries also abolished acetylcholine-mediated relaxation but had no effect on phenylephrine or ET-1 (Figure S3; Table S2). SMET_B KO had no effect on contractile responses to phenylephrine or ET-1, or acetylcholine-mediated relaxation in femoral arteries (Figure S4; Table S3).

Induction of ET_B-Mediated Contraction in Isolated Mesenteric Arteries

ET-1-mediated contraction in mesenteric arteries from WT C57Bl/6J mice was shifted to the right by mixed ET_{A/B}, or selective ET_A, antagonism, but not by ET_B selective antagonism (Figure S5; Table S4). Unlike mesenteric veins (Figure 6A), mesenteric arteries freshly isolated from WT C57Bl/6J mice did not contract in response to S6c (Figure 6B).

Incubation in culture medium (\leq 5 days) can induce ET_B-mediated contraction in rat arteries.²⁹ Incubation of C57Bl/6J mesenteric veins in culture medium had no effect on S6c-mediated contraction (Figure 6A). In mesenteric arteries, incubation in culture medium selectively increased the contractile response to ET-1 (Table S5). Strikingly, S6c-mediated contraction was induced in isolated mesenteric arteries after incubation in culture medium (Figure 6B; Table S5), a response abolished by selective ET_B, or mixed ET_{A/B}, antagonism, but not by selective ET_A antagonism (Figure 6C; Table S6). Incubation of mesenteric arteries from SMET_B KO mice in culture medium did not induce S6c-mediated contraction (Figure 6D).

No Induction of ET_B-Mediated Contraction in Femoral Arteries

S6c-mediated contraction was variable in femoral arteries from WT C57Bl/6J mice: some contracted but others did not (Figure 6E). Neither incubation of femoral arteries in culture medium (24 hours; Figure 6F) nor lesion formation induced S6c-mediated contraction; femoral arteries isolated 28 days after ligation contracted in response to ET-1 (Figure 6G) but not to S6c (Figure 6H). Responses to acetylcholine, sodium

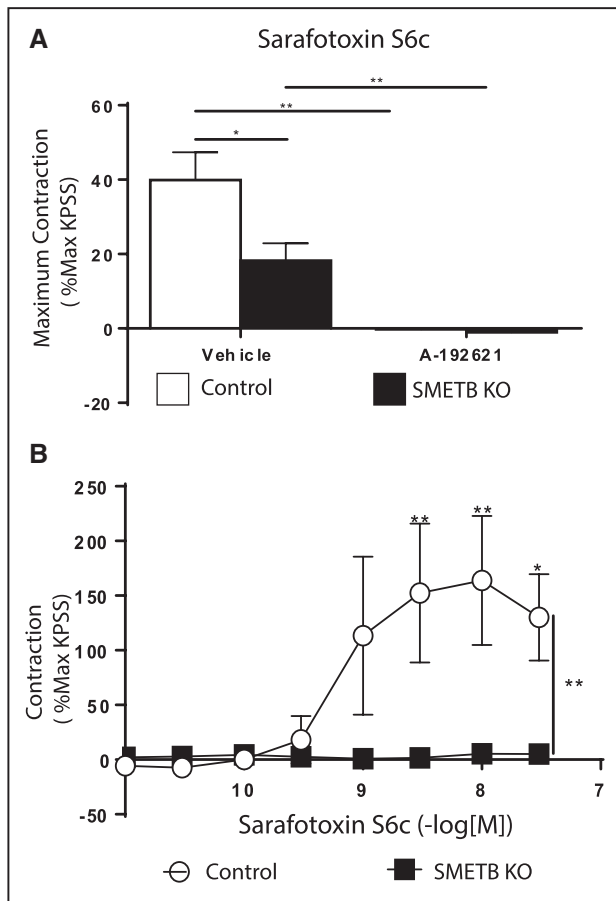


Figure 2. Functional consequences of selective endothelin_B (ET_B) deletion from smooth muscle (SM). **A**, Sarafotoxin S6c (S6c)-induced contraction of isolated trachea was abolished by ET_B receptor antagonism (A192621; 100 nmol/L) but only reduced by selective smooth muscle ET_B receptor (SMET_B) deletion (residual contraction was blocked by A192621). Columns are mean \pm SEM (n=4). **P*<0.02, ***P*<0.005. **B**, S6c-induced contraction in murine mesenteric veins was abolished by SMET_B deletion. Symbols represent mean \pm SEM (n=4). **P*<0.05, ***P*<0.01. KO indicates knockout; and KPSS, potassium physiological salt solution.

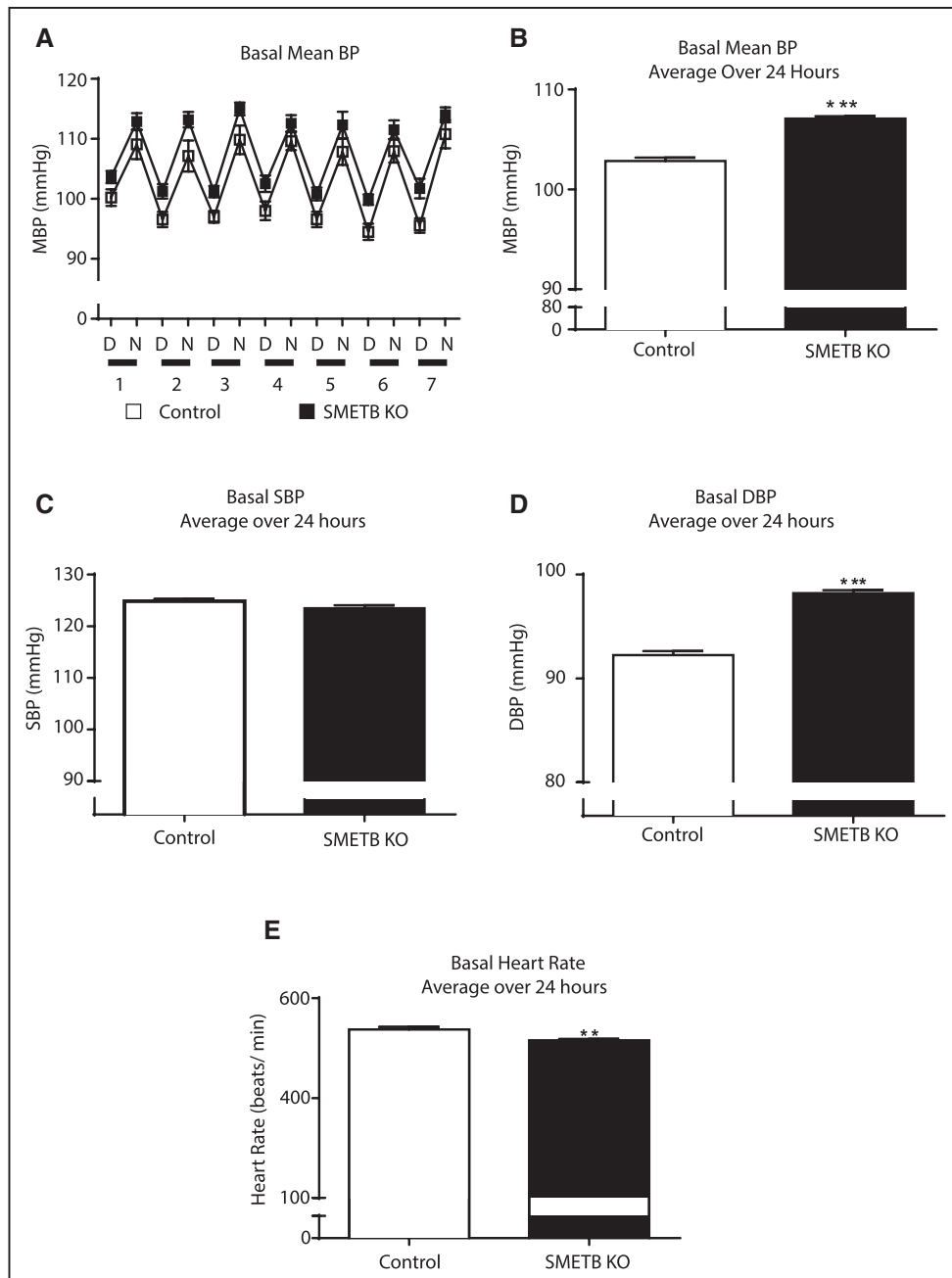


Figure 3. Selective deletion of endothelin_B (ET_B) receptors from smooth muscle increases baseline blood pressure (BP). **A**, BP, assessed in conscious, unrestrained male smooth muscle ET_B receptor knockout (SMET_B KO) mice and controls (n=8 per group) using radiotelemetry, demonstrated a clear diurnal rhythm. Mean blood pressure (MBP) in SMET_B KO (filled symbols) mice was consistently higher than controls (open symbols). **B**, Data averaged over 24 h confirmed elevated MBP in SMET_B KO, with no difference in **(C)** systolic blood pressure (SBP) but **(D)** elevated diastolic blood pressure (DBP). **E**, Increased MBP was accompanied by reduced heart rate. Data are mean±SEM (n=8 per group). ***P*<0.005, ****P*<0.0001. D indicates day; and N, night.

nitroprusside, and phenylephrine were unaltered by lesion formation (Figure S6).

Discussion

Tissue-specific knockout mice were generated to address the hypothesis that selective deletion of ET_B receptors from VSMC would impair arterial contraction, lower BP, and reduce neointimal lesion size. SMET_B KO attenuated S6c-mediated vascular and tracheal contraction, without altering other functional responses, but produced a modest (≈4

mm Hg) increase in BP. ET_B-mediated contraction was not induced in femoral arteries after ligation, although injury-induced intimal lesion formation was unaffected by SMET_B KO. Key findings are summarized (Figure S7) and compared with the ECET_B KO (Table S7).

SMET_B KO was based on our generation of ECET_B KO,²² crossing mice expressing Cre-recombinase controlled by the SM-specific SM22 promoter²⁵ with those bearing a floxed ET_B gene.²² This strategy was used to produce mice with SM-selective ET_A deletion,⁴ and renal collecting duct-selective

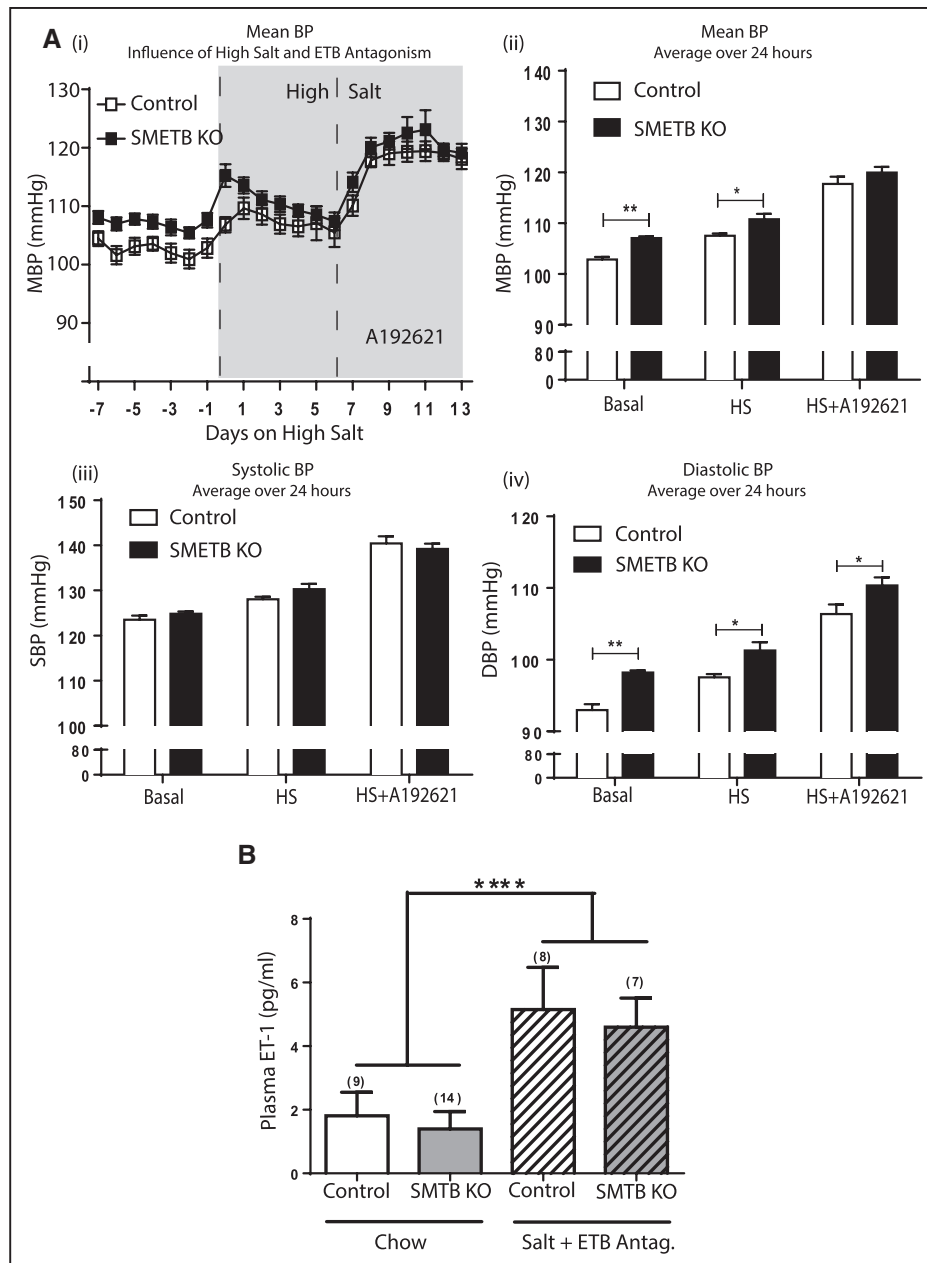


Figure 4. Selective deletion of endothelin_B (ET_B) receptors from smooth muscle does not alter blood pressure (BP) responses. **A**, BP, assessed in conscious, unrestrained male smooth muscle ET_B receptor knockout (SMET_B KO) mice and controls ($n=8$ per group) using radiotelemetry (i) was elevated by high salt diet (HS; 7 d) and by ET_B antagonism (A192621; $30 \text{ mg}^{-1} \text{ kg}^{-1} \text{ d}^{-1}$; 7 d) in both groups. (ii) Comparison of BP (averaged over 24 h) demonstrates the elevation in mean blood pressure (MBP) in response to high salt diet and high salt diet plus A192621. (iii) There was no difference in systolic blood pressure (SBP) in control compared with SMET_B KO mice but (iv) diastolic blood pressure (DBP) was higher in SMET_B KO for all treatment groups. **B**, Plasma endothelin-1 (ET-1) concentrations were similar in SMET_B KO and controls and consistent with wild-type C57Bl/6J mice ($1.14 \pm 0.08 \text{ pg/mL}$; $n=6$). ET-1 concentrations were elevated in control and SMET_B KO mice after exposure to a high salt diet plus A192621. Data (mean \pm SEM) were analyzed using 2-way ANOVA with Tukey or Bonferroni post hoc test, as appropriate. **A**, * $P<0.05$, ** $P<0.01$ compared with controls. **B**, **** $P<0.00001$ (effect of diet).

ET_B deletion.²¹ It has also been used within our group to produce mice with SM-selective deletion of glucocorticoid receptor³¹ or 11 β -hydroxysteroid dehydrogenase 1³² (with LacZ staining in Rosa26 reporter mice showing SM22cre expression in the blood vessels and heart but not in the brain, kidney, or adrenal gland). As with ECET_B KO,²² SMET_B KO mice were healthy. This contrasts with global ET_B deletion, which causes coat spotting and death from megacolon,³³ requiring transgenic ET_B rescue in the enteric nervous system.³⁴ Autoradiographic

detection of ET_B receptors in lungs of SMET_B KO mice indicates maintained expression in EC (which was lost in ECET_B KO).¹⁴ This was supported by colocalization of immunoreactivity for ET_B with an EC marker (von Willebrand factor) in coronary arteries; absence of medial ET_B staining was consistent with deletion from SMCs. Polymerase chain reaction confirmed that ET_B had been deleted from aortic smooth muscle but not from heart, colon, or skeletal muscle (although direct evidence of ET_B deletion from tracheal, mesenteric vein,

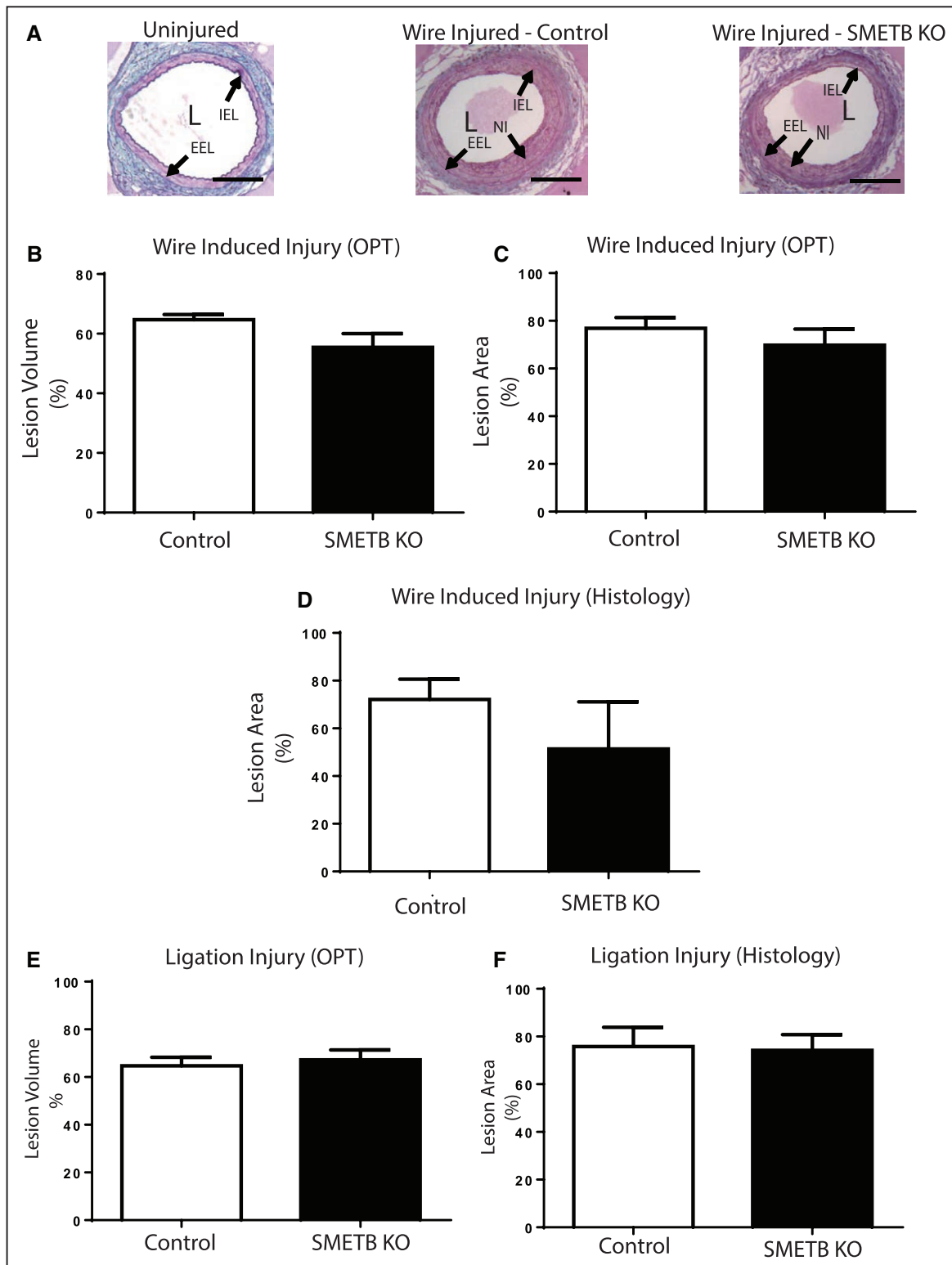


Figure 5. Selective smooth muscle endothelin_B (SMET_B) deletion does not alter neointimal lesion formation. **A**, Wire injury–induced lesion formation in femoral arteries from control and SMET_B knockout (KO) mice. Neointimal lesion volume (**B**) and maximal cross-sectional area (**C**) were similar in control and SMET_B KO mice when measured by optical projection tomography. Similar results were obtained when maximal cross-sectional area was measured histologically (**D**). Volume (**E**) and maximal cross-sectional area (**F**) of lesions induced by ligation were similar in control and SMET_B KO mice (optical projection tomography [OPT]). Data are mean±SEM (n=7). EEL indicates external elastic lamina; IEL, internal elastic lamina; L, lumen; and NI, neointima.

mesenteric, or femoral artery smooth muscle was not obtained using this technique). Functional investigations confirmed that SMET_B-dependent responses were lost in the knockout,

with the abolition of S6c-mediated contraction in mesenteric veins. Furthermore, induction of S6c-mediated contraction in mesenteric arteries incubated in culture medium (as in rat

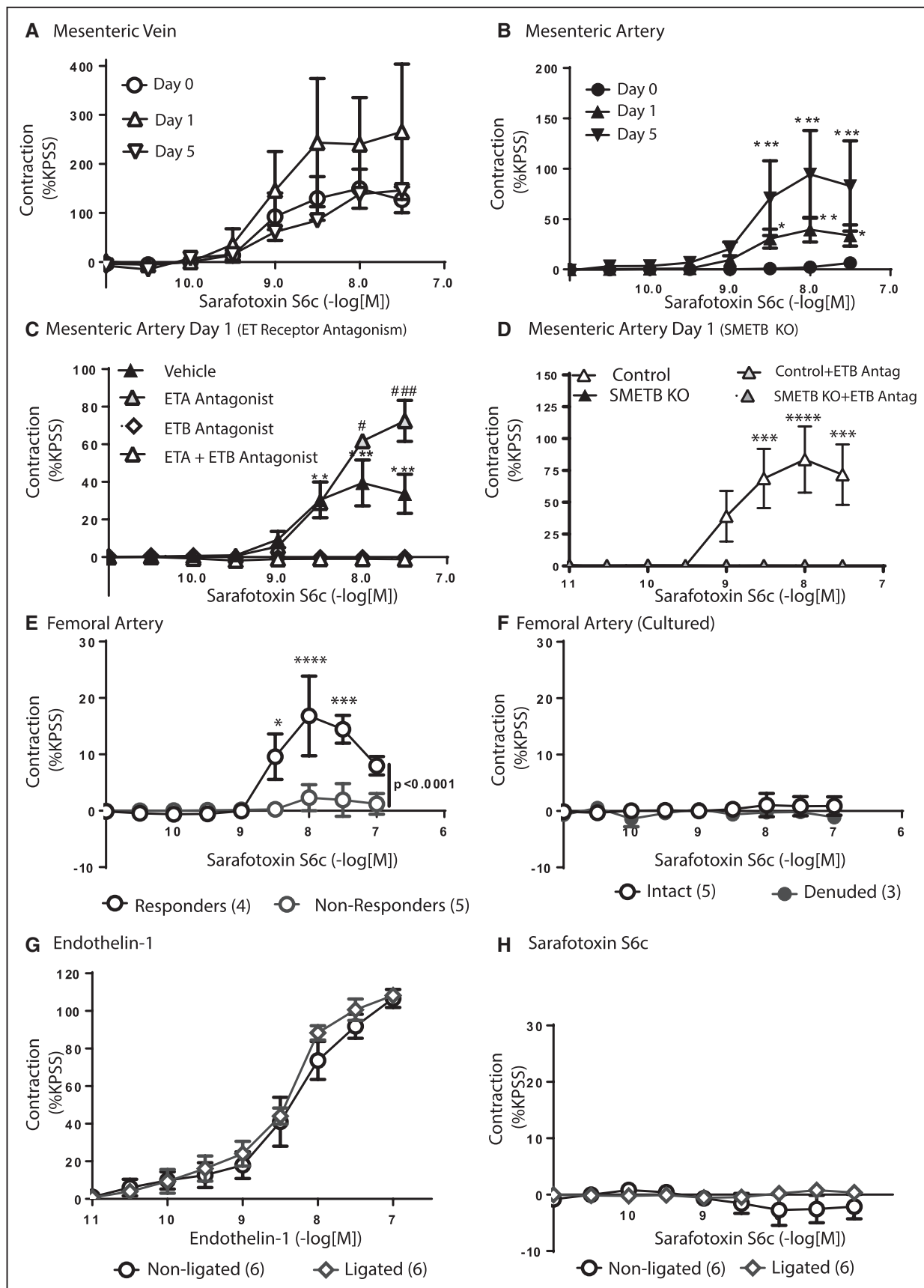


Figure 6. Impact of smooth muscle endothelin_B (SMET_B) receptors on vascular function. **A**, Sarafotoxin S6c (S6c)-induced contraction in mesenteric veins (n=6) was not increased by incubation for 1 (n=3) or 5 (n=1) d in culture. **B**, Freshly isolated mesenteric arteries (n=6) did not respond to S6c, but contractions were induced by incubation in culture medium for 1 (n=7) or 5 (n=3) *P<0.05, (Continued)

Figure 6 Continued. ** $P < 0.01$, *** $P < 0.005$ compared with d 0. **C**, S6c-mediated contraction of mesenteric arteries after 24 h in culture ($n = 7$) was abolished by ET_B selective (A192621; 100 nmol/L; $n = 3$) or mixed ET_{A/B} (BQ-123+A192621; $n = 3$) antagonism, but not by ET_A receptor antagonism (BQ-123; 100 nmol/L; $n = 3$); ** $P < 0.01$, *** $P < 0.005$ compared with ET_B or ET_{A/B} antagonism; # $P < 0.05$, ### $P < 0.005$ compared with vehicle. **D**, In contrast to controls ($n = 4$), S6c-mediated, A192621 (100 nmol/L)-sensitive contraction was not induced in mesenteric arteries from SMET_B knockout (KO) mice ($n = 4$) by incubation in culture medium (24 h); *** $P < 0.005$, **** $P < 0.001$ compared with antagonists. **E**, Contractile responses to S6c were unreliable in femoral arteries—some failed to contract, whereas others produced small contractions. * $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$ compared with nonresponders. **F**, Incubation in culture did not induce S6c-mediated contraction in these arteries. Femoral arteries after ligation (28 d) contracted in response to endothelin-1 (**G**) but not to S6c (**H**). Data are mean \pm SEM ($n = 3$ to 6). ET_A indicates endothelin_A; and KPSS, potassium physiological salt solution.

arteries³⁵), was abolished by SMET_B KO (although these functional changes do not necessarily confirm selective SMET_B deletion). The failure to abolish S6c-induced contraction in trachea was unexpected and suggests either incomplete penetrance of SM22cre-mediated recombination or a role for ET_B receptors in other cells (eg, epithelium) in mediating tracheal contraction. Detection of the delta band in some ear clip samples may suggest deletion of the floxed gene in germ cells, which is a possible limitation with these mice. However, our F⁺/Cre0x⁺/Cre0 crosses did not produce piebald mice (which inevitably would occur if germ-line recombination takes place). Therefore, the delta band during genotyping can only be explained by the presence of SMC in the ear clip preparations.

Selective deletion of ET_B from EC increased plasma ET-1²² because of impaired clearance.¹⁴ In contrast, SMET_B KO did not alter circulating ET-1, consistent with the proposal that ECET_B predominantly mediate ET-1 clearance.

Transgenic and pharmacological approaches suggest ET_B receptors regulate BP. Selective ET_B receptor antagonism,²⁰ global ET_B deletion,¹⁰ and selective ET_B deletion from the collecting duct²¹ all increased (≈ 10 – 13 mm Hg) BP. Furthermore, ET_B receptors in peripheral ganglia can influence BP,³⁶ suggesting that sympathetic activation accounts for ET_B-induced hypertension.³⁷ In contrast, BP was not elevated by ECET_B KO.²² The small (≈ 4 mm Hg) increase in BP, which persisted in SMET_B KO mice despite reduced heart rate, suggests that loss of SMET_B contributes to the increased BP induced by systemic ET_B antagonism²⁰ or global ET_B deletion.¹⁰ However, it requires rejection of our hypothesis that ET_B-mediated vascular contraction contributes to BP elevation. Indeed, our data support a role for extravascular ET_B (eg, in the kidney or peripheral ganglia) in regulating BP. This is supported by the demonstration that, as in ECET_B KO,²² salt-induced and ET_B antagonist-induced elevations of BP are unaltered by SMET_B KO. The mechanism underlying increased BP after SMET_B KO is not apparent but is unlikely to be a consequence of cre overexpression in SM because this did not alter baseline BP in SMET_A KO mice.⁴ Several possible explanations can be proposed. First, ET_B in VSMC may contribute to the clearance of ET-1 from tissue where it is preferentially secreted by EC, and where it acts. Therefore, SMET_B KO may cause ET-1 accumulation in the vascular wall, thus increasing ET-1-mediated vasoconstriction. Second, loss of SMET_B may upregulate ET_A-mediated contraction. Third, SMET_B in the kidney may influence sodium homeostasis. Because SM22 may be expressed in perivascular fat precursors,³⁶ loss of ET_B from perivascular fat may have caused developmental changes in vascular function that also contribute to elevated BP, but this has not been established. It is also not clear why basal diastolic blood

pressure is selectively increased in the SMET_B KO, but this would be worthy of future investigation.

Increased BP in SMET_B KO mice could not be attributed to vascular dysfunction as, with the exception of responses to S6c, we found no evidence of impaired arterial relaxation or contraction. Weak ET_B-mediated contraction in arteries is consistent with studies in rats.³⁵ Preliminary investigations (unpublished data) indicated that S6c-induced contraction of freshly isolated murine arteries (femoral, mesenteric, and carotid) was not increased by NO synthase inhibition or by removal of the endothelium. These results indicate that we are not missing an ET_B-mediated contraction that has been obscured by ET_B-mediated relaxation. Induction of ET_B-mediated contraction after incubation has been attributed to transcriptional regulation and MEK-ERK1/2 signaling.^{22,38} Abolition of this response in mesenteric arteries from SMET_B KO mice indicated that they lack both functional arterial ET_B receptors and the means to generate new receptors in this tissue.

ET_B upregulation in SMC, mediating vasoconstriction and proliferation in cardiovascular disease,^{18,19} might explain studies reporting similar benefit from mixed ET_{A/B} and selective ET_A antagonism in reducing lesion formation^{23,39,40} (despite the protective roles of ET_B in several tissues, eg, EC and kidney). However, the effectiveness of mixed ET_{A/B} and selective ET_A antagonism is likely to depend on the balance of ET_B receptor activity in EC and VSMC of an affected artery. Transient upregulation of ET_A and ET_B receptors has been demonstrated in arterial lesions.⁴¹ If these ET_B receptors contribute to lesion formation, then ET_B antagonism would be desirable. There was, however, no evidence of induced ET_B-mediated contraction in mouse femoral arteries after ligation. Similar investigations could not be performed after wire injury because these vessels fail to contract *ex vivo*. It remains possible that ET_B upregulation occurs in other (eg, carotid) arteries.

Neointimal lesion formation is increased in rescued global ET_B knockout mice¹⁰ and in (spotted lethal) rats with global deletion of ET_B,²⁴ consistent an antiproliferative role for ET_B receptors. This is supported by demonstrations that ET_B receptor antagonism increases lesion size,^{9,24} with the suggestion that this is because of impaired ET_B-mediated release of NO from EC. Indeed, increased lesion formation in mice with global ET_B deletion was partly attributed to impaired EC-derived NO release.⁹ In contrast, selective ECET_B deletion inhibited ET_B-mediated relaxation²² but had no effect on arterial lesion formation.⁹ These results suggest, therefore, that the protective role of ET_B receptors is played by non-ECET_B receptors. The demonstration here that deletion of ET_B from the SMC does not alter lesion size indicates that, as with the receptors in EC,⁹ ET_B in SMC do not influence neointimal remodeling. This implicates nonvascular ET_B receptors, for

example, in monocyte-derived macrophages, in the regulation of neointimal proliferation and atherosclerosis.⁴²

In conclusion, we have demonstrated that selective ET_B receptors in SMC may contribute modestly to regulation of BP but have little influence on vascular contraction or neointimal proliferation. These data suggest that any detrimental role of SMET_B is minor (at least during normal physiology), and, therefore, that selective ET_A receptor antagonists (which preserve protective EC/renal ET_B signaling) should be preferred to mixed ET_{A/B} antagonists for treatment of vascular disease.

Perspectives

Generation of mice with selective deletion of ET_B from SMC indicates that these receptors contribute to the increased BP induced by ET_B receptor antagonism but do not regulate arterial function or the fibroproliferative response to acute arterial injury. It would be interesting to determine whether ET_B in SMCs influence other cardiovascular diseases (eg, diabetic complications). Whether the data generated in these animals are replicated in mice with cardiovascular disease (eg, atherosclerosis), or in man, remains to be established. However, these results support the proposal that selective ET_A receptor antagonists may have advantages over mixed ET_{A/B} antagonists for combatting elevated BP or restenosis after revascularization.

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Disclosures

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Novelty and Significance

What Is New?

- This study describes newly generated mice with selective endothelin_B (ET_B) receptor deletion from smooth muscle. This was used to clarify the influence of smooth muscle ET_B receptors on (1) blood pressure, (2) arterial and venous contraction, and (3) arterial remodeling following injury.

What Is Relevant?

- Generation of the knockout was necessary because ET_B receptors in vascular endothelial and smooth muscle cells cannot be distinguished pharmacologically. This work shows that ET_B receptors in smooth muscle have little influence on arterial function or neointimal remodeling but have a

small suppressive effect on diastolic blood pressure. This is consistent with the proposal that selective endothelin_A (ET_A) antagonism would be preferable to mixed ET_A/ET_B antagonism for inhibiting arterial remodeling.

Summary

Selective smooth muscle ET_B deletion indicated that these receptors play a minor role in regulation of BP but do not affect vascular function or remodeling. This suggests that, beyond endothelial cell ET_B, ET_B-dependent regulation of these processes is mediated by receptors in extravascular cells (eg, renal collecting ducts).

Smooth Muscle Endothelin B Receptors Regulate Blood Pressure but Not Vascular Function or Neointimal Remodeling

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SMOOTH MUSCLE ENDOTHELIN B RECEPTORS REGULATE BLOOD PRESSURE
BUT NOT VASCULAR FUNCTION OR NEOINTIMAL REMODELLING

Supplementary Information

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Short Title: ET_B deletion, BP and arterial remodelling

Animals

Mice with VSMC selective deletion of ET_B receptors were generated based on a strategy described previously to produce EC ET_B KO mice¹. Homozygous (Flox/Flox) ET_B mice (background: 50% 129/Ola and 50% BKW) were crossed with SM22-Cre transgenic mice and backcrossed to C57Bl/6J for 4-6 generations. As for previous studies^{1,2} floxed, Cre-negative littermates (ET_B^{f/f}) were used as experimental controls for SM-specific ET_B deficient mice (SM ET_B KO). Genotyping to identify wild type and recombined alleles was performed by PCR¹ and the SM22-Cre transgene was detected as described³. All mice were given free access to tap water and standard mouse chow. Mice were housed according to United Kingdom Home Office recommendations at 22°C with 12-hour diurnal light/dark cycles. All procedures were performed under the provisions of the Animals Scientific Procedures Act (1986) and with the approval of the local ethics committee.

Autoradiography

After euthanasia, SM ET_B KO mice and controls (n=3/group) were rapidly frozen at -70°C, torsos were mounted in a cryostat and consecutive 30µm longitudinal sections were cut to encompass the major thoracic and abdominal organs. Sections were thaw-mounted onto gelatin-coated slides⁴ and ligand binding assays performed as described⁵. Briefly, consecutive sections were incubated with 0.25nM of the ET_A selective ligand [¹²⁵I]PD151242 or 0.25nM of the ET_B selective ligand [¹²⁵I]BQ2030 (Amersham Bioscience, GE Healthcare, UK). Non-specific binding was determined by co-incubating adjacent sections with the ligand and corresponding excess unlabelled peptide. Slides with calibrated standards were exposed to Kodak MR-1 autoradiography film for 4 days before being developed.

Immunohistochemistry

Immunohistochemical identification of ET_B receptors was performed as previously described⁶. Site directed antisera were raised in rabbits to the sequence ET_B(302-313), as described⁷. Briefly, whole body tissue sections (15 µm) were dried overnight at room temperature and fixed in ice-cold acetone for 10 minutes. Three knock-out and three control mice were examined.

Slides were incubated with 5% non-immunised donkey serum (DS) in phosphate-buffered saline (PBS) for 1 hour at room temperature to block non-specific protein interactions and then incubated overnight at 4 °C with primary rabbit anti-ET_B (1:50) antiserum and primary goat anti-von Willebrand factor (1:50) PBS/0.1% Tween-20/3% DS. Slides were then washed (3×5 minutes) in cold 1% PBS/0.1% Tween-20 before incubation for 1 hour at room temperature with Alexa Fluor 488 conjugated donkey anti-rabbit (1:200), Alexa Fluor 568 conjugated donkey anti-goat (1:100) secondary antibodies and Hoechst (1:100) diluted in 1% PBS/0.1% Tween-20/3% DS. Tissue sections were washed again (3×5 minutes) in cold 1% PBS/0.1% Tween-20 and mounted with ProLong Gold (Invitrogen). Confocal imaging was performed using a Leica TCS-NT-UV confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

Ex vivo analysis of ET_B-mediated contraction

Functional analyses were performed using isolated mouse trachea, aorta, femoral arteries, and 1st order mesenteric arteries and veins, as described⁸. The endothelium was removed from some aortic rings by rubbing the luminal surface with a wire. Some arteries and veins were incubated in serum-free medium (DMEM) for 1-5 days before functional analysis to induce ET_B-mediated contraction, after the method of Adner *et al.*⁹. Briefly, rings (~2mm in length) of trachea, femoral artery, or mesenteric artery or vein were suspended on two intraluminal

40 μm tungsten wires in a myograph (model 610M Multi-myograph; JP Trading, Aarhus, Denmark) chamber. These rings were equilibrated at their optimum resting force (trachea 2mN; aorta 7.36mN, femoral artery, 8mN; mesenteric artery 3mN; mesenteric vein, 1mN) in physiological salt solution (PSS; 119 mM NaCl, 14.9 mM NaHCO_3 , 4.7 mM KCl, 1.18 mM KH_2PO_4 , 1.17 mM MgSO_4 , 1.6 mM CaCl_2 , 0.026 mM EDTA, 5.5 mM glucose), aerated (95% O_2 , 5% CO_2) and maintained at 37°C. Each ring was then exposed to a high potassium (125mM) PSS (KPSS). Cumulative concentration-response curves were obtained to phenylephrine (PE, 1×10^{-9} - 3×10^{-3} M), ET-1 (1×10^{-11} - 3×10^{-5} M), acetylcholine (1×10^{-9} - 3×10^{-3} M) or sarafotoxin 6c (S6c; 1×10^{-11} - 3×10^{-5} M), as required. Some rings were incubated with an ET_A antagonist (BQ123; 100nM), an ET_B antagonist (A192621; 100nM) or a mixture of the two antagonists 20 min before acquiring cumulative concentration-response curves. All responses were measured and recorded with Powerlab software.

Measurement of BP

Male SM ET_B KO mice and age-matched controls (n=8/ group) were caged singly and maintained on standard mouse chow (7 days) before measurement of BP and heart rate using telemetry, as described previously¹. Briefly, under isoflurane anesthesia, a telemetry catheter was inserted into the left carotid artery and the transmitter device (Data Sciences) secured in the left flank. Mice were allowed to recover and were maintained on standard chow (7 days), high (7.6%) salt diet (7 days), then high salt plus ET_B antagonist (SB192621; 30/mg/kg/day in drinking water, 7 days). Systolic and diastolic BP and heart rate were recorded in unrestrained mice (for 5 min every 30 min) as described previously¹ and analyzed using the Powerlab data acquisition system. Average blood pressures over each 5 min period (48 measurements/ day) were used to calculate the 24 h average BP.

Femoral artery injury

Intra-luminal injury was performed as described². Briefly, under inhaled isoflurane anaesthesia (induction 5%, maintenance 2-3%) a 0.014" diameter straight-sprung angioplasty guide wire was advanced ~1.5cm proximally into the isolated femoral artery through an arteriotomy in the popliteal branch. After withdrawal, the popliteal branch was ligated to allow re-perfusion of the injured femoral artery. Non-denuding injury was achieved by ligation of the right femoral artery at the femero-popliteal bifurcation². Peri-operative analgesia was provided by administration of buprenorphine (0.05mg/kg buprenorphine s.c.; Alstoe Animal Health, UK). Mice were then allowed to recover (28 days) to allow lesion development.

Perfusion fixation

After the recovery period mice were killed by perfusion fixation. Under terminal anaesthesia (sodium pentobarbital, Ceva Animal Health, UK, 60 mg/kg; i.p.), thoracotomy and transverse sternotomy were performed to allow introduction of a 23-gauge needle into the left ventricle. Phosphate buffered saline containing heparin (Leo Laboratories, UK, 10 U/ml) was administered (6 ml/min) via the left ventricle and an incision was made in the right ventricle to allow perfusate to wash through. Once blood was washed out, 10% neutral buffered formalin (Sigma, UK) was perfused until adequate fixation occurred (indicated by the development of rigidity of the body). Following perfusion fixation, femoral arteries, liver, heart and kidneys were removed. Organs were weighed and all tissues were left in formalin for a further 48h before processing to paraffin for histological assessment.

Optical projection tomography

Non-destructive 3-dimensional assessment of lesions was performed using optical projection tomography (OPT), as described^{10,11}. Briefly, vessels were embedded in agarose and optically cleared in benzyl alcohol/ benzyl benzoate. Intrinsic fluorescent emission was imaged (excitation filter: 425/40 nm; emission filter: 475 nm low pass) using a Bioptonics 3001 tomograph. Data were reconstructed by filtered back projection using NRecon software (Skyscan, Belgium) and volumetric measurements generated by semi-automated tracing of the internal elastic lamina and the neointima distinguished from the lumen using a grey level threshold.

Histological assessment of neointimal lesions

Sections (4 µm) were cut from paraffin-embedded femoral arteries at 80 µm intervals with a Leitz 1512 microtome (Leica microsystems, Germany), and mounted onto Superfrost glass slides. Every tenth slide was selected for staining (Shandon Varistain Gemini automated slide stainer) with United States Trichrome, as described¹². Images were taken using an Axioskop KS300 stage microscope (Carl Zeiss Inc., UK) and a CCD camera (photometrics USA) with a liquid crystal filter (MicroColour, LRI, Inc, USA). Image analysis was performed using MCID basic 7.0 software (Imaging Research, USA). The location of the maximal lesion was determined and serial sections used for compositional analysis, including picro-sirius staining for the quantification of collagen content.

Immunohistochemistry

De-waxed and re-hydrated sections were blocked with goat serum before incubation with primary antibodies to α -smooth muscle actin (1:400; 30 min; Sigma, UK) and Mac2 (1:6000; overnight; Cedarlane, USA). Sections were then washed and incubated with a secondary antibody (goat anti-mouse or goat anti-rat, respectively; 1:400, 30 min; Vector Labs, UK). This was followed by incubation with streptavidin-conjugated horseradish peroxidase Extravidin; 30 min; Sigma, UK). Slides were developed by addition of 3,3-diaminobenzidine (DAB peroxidase staining kit, Vector Lab, UK) for 1 min. Images were taken as before and analysed with Image J software.

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Supplementary Table S1. Deletion of ET_B from smooth muscle did not alter body or organ weights.

Age & Weights	Wild Type	SMET_B KO
Age (weeks)	20.0 ± 1.4	20.8 ± 1.6
Body Weight (g)	31.6 ± 0.8	32.1 ± 0.6
Heart (% Body Weight)	0.56 ± 0.03	0.56 ± 0.02
Liver (% Body Weight)	5.08 ± 0.15	4.88 ± 0.18
Right Kidney (% Body Weight)	0.72 ± 0.03	0.74 ± 0.03
Right Kidney (% Body Weight)	0.68 ± 0.02	0.71 ± 0.03
Lung (% Body Weight)	0.54 ± 0.03	0.56 ± 0.02

Data are mean ± s.e. mean, n=7.

Supplementary Table S2. Impact of endothelial cell removal on function of aorta and femoral arteries from control mice.

Drug	Measurement	Aorta (Intact)	Aorta (Denuded)	Femoral Artery (Intact)	Femoral Artery (Denuded)
PE	N	3	3	3	3
	pD ₂	7.1 ± 0.2	7.3 ± 0.2	5.8 ± 0.1	6.5 ± 0.1*
	E _{max} (%)	80.3 ± 5.5	199.8 ± 11.4*	100.3 ± 6.2	94.6 ± 4.5
	E _{max} (mN/mm)	3.92 ± 0.34	3.48 ± 0.19	2.35 ± 0.20	1.61 ± 0.14
ACh	N	3	3	3	3
	-logIC ₅₀	7.4 ± 0.4	7.9 ± 1.0	7.4 ± 0.2	6.5 ± 6.1
	E _{max} (%)	62.7 ± 7.1	31.5 ± 7.3	108.6 ± 5.7	19.5 ± 11.8*
ET-1	N	3	3	3	3
	pD ₂	8.5 ± 0.4	8.0 ± 0.2	8.2 ± 0.2	8.4 ± 0.1
	E _{max} (%)	53.4 ± 8.4	74.3 ± 4.7	95.2 ± 4.5	89.6 ± 4.2
	E _{max} (mN/mm)	1.78 ± 0.42	1.23 ± 0.20	2.51 ± 0.36	2.35 ± 0.46
KPSS	N	4	4	4	4
	E _{max} (mN/mm)	2.91 ± 0.58	2.03 ± 0.43	3.21 ± 1.36	2.56 ± 1.23

Data are mean±s.e.mean. ACh, acetylcholine, ET-1, endothelin-1, KPSS, high (125mM) potassium physiological salt solution. PE, phenylephrine. *P<0.05 compared with intact artery. E_{max}, maximum contraction; pD₂, -log EC₅₀.

Supplementary Table S3 Impact of deletion of ET_B from vascular smooth muscle on functional responses of femoral arteries.

Drug	Measurement	Wild Type (Intact)	Wild Type (Denuded)	SM ET _B KO (Intact)	SM ET _B KO (Denuded)
PE	N	6	7	7	7
	pD ₂	5.7 ± 0.1	6.0 ± 0.1	5.6 ± 0.1	6.1 ± 0.1
	E _{max} (%)	88.9 ± 3.2	94.2 ± 3.2	91.9 ± 4.4	102.8 ± 3.8
	E _{max} (mN/mm)	3.99 ± 0.35	2.05 ± 0.26	3.48 ± 0.25	2.37 ± 0.20
ACh	N	5	6	6	6
	-logIC ₅₀	8.0 ± 0.6	6.8 ± 0.4 [†]	7.9 ± 0.5	7.2 ± 0.6*
	E _{max} (%)	120.1 ± 10.2	57.8 ± 6.4*	120.9 ± 9.6	22.5 ± 3.2 [‡]
ET-1	N	6	7	7	7
	pD ₂	7.8 ± 0.2	7.8 ± 0.3	7.9 ± 0.2	8.0 ± 0.2
	E _{max} (%)	97.4 ± 7.9	112.9 ± 3.5	105.4 ± 5.8	130.3 ± 5.4
	E _{max} (mN/mm)	4.03 ± 0.42	2.46 ± 0.40	4.04 ± 0.33	2.99 ± 0.27
KPSS	N	6	7	7	7
	E _{max} (mN/mm)	4.44 ± 0.81	1.99 ± 0.64*	3.84 ± 0.52	2.38 ± 0.54

Data are mean±s.e.mean. SM ET_B KO, selective deletion of the endothelin B receptor from smooth muscle. ACh, acetylcholine, ET-1, endothelin-1, KPSS, high (125mM) potassium physiological salt solution. PE, phenylephrine. *P<0.05, [†]P<0.01, [‡]P<0.005 compared with intact artery. E_{max}, maximum contraction; pD₂, -log EC₅₀.

Supplementary Table S4. Impact of endothelin receptor antagonism on endothelin-1-mediated contraction of murine mesenteric arteries.

Measurement	Vehicle	Antagonist		
		ET _A	ET _B	ET _{A/B}
N	7	3	3	3
pD ₂	8.2±0.5	7.4±0.2*	8.0±0.1	7.0±0.1*
E _{max} (mN/mm)	2.4±0.7	2.4±1.3	3.4±0.5	2.3±1.0
E _{max} (% KPSS)	105±17	102±21	111±10	75±30

Data are mean±s.e.mean. *P<0.05 compared with Vehicle. E_{max}, maximum contraction; pD₂, -log EC₅₀.

Supplementary Table S5. Incubation induces ET_B-mediated contraction in mouse mesenteric arteries.

Drug	Measurement	Incubation		
		Day 0	Day 1	Day 5
KPSS	N	7	7	3
	E _{max} (mN/mm)	2.47±0.80	2.42±0.68	1.40±1.03
PE	N	7	7	3
	pD ₂	5.90±0.25	5.71±0.24	5.65±0.44
	E _{max} (mN/mm)	2.33±0.70	2.89±1.30	1.04±1.24
	E _{max} (% KPSS)	97.8±15.6	114.1±25.1	111.7±91.7
S6c	N	7	7	3
	pD ₂	---	8.75±0.19	8.73±0.05
	E _{max} (mN/mm)	0.13±0.16	1.13±1.08*	0.82±0.60 [†]
	E _{max} (% KPSS)	6.4±7.6	40.2±32.6*	94.5±75.4 [†]
ET-1	N	7	5	3
	pD ₂	8.24±0.53	8.50±0.40	8.54±0.25
	E _{max} (mN/mm)	2.45±0.73	3.79±0.88*	1.35±1.22*
	E _{max} (% KPSS)	105.4±17.8	164.1±16.4*	132.8±87.6

Data are mean±s.e.mean.. ET-1, endothelin-1, KPSS, high (125mM) potassium physiological salt solution, PE, phenylephrine, S6c, sarafotoxin s6c. *P<0.05 compared with Day 0.

[†]P<0.05 compared with Day 1. E_{max}, maximum contraction; pD₂, -log EC₅₀.

Supplementary Table S6. Impact of endothelin receptor antagonism on sarafotoxin s6c-mediated contraction of murine mesenteric arteries.

Timepoint	Measurement	Vehicle	Antagonist		
			ET _A	ET _B	ET _{A/B}
Day 0	N	7	3	3	3
	pD ₂	NC	NC	NC	NC
	E _{max} (mN/mm)	0.13±0.16	0.01±0.01	0.03±0.03	0.04±0.04
	E _{max} (% KPSS)	6.4±7.6	0.90±1.3	1.0±0.6	1.2±1.2
Day 1	N	7	3	3	3
	pD ₂	8.75±0.19	8.41±0.17*	NC	NC
	E _{max} (mN/mm)	1.13±1.08	2.63±0.90	0.01±0.01	0.00±0.01
	E _{max} (% KPSS)	40.2±32.6	73.3±17.75	0.7±0.4*	0.4±0.4*

Day 0, fresh arteries; Day 1, 24 h incubation. Data are mean±s.e.mean.. NC, not calculated.

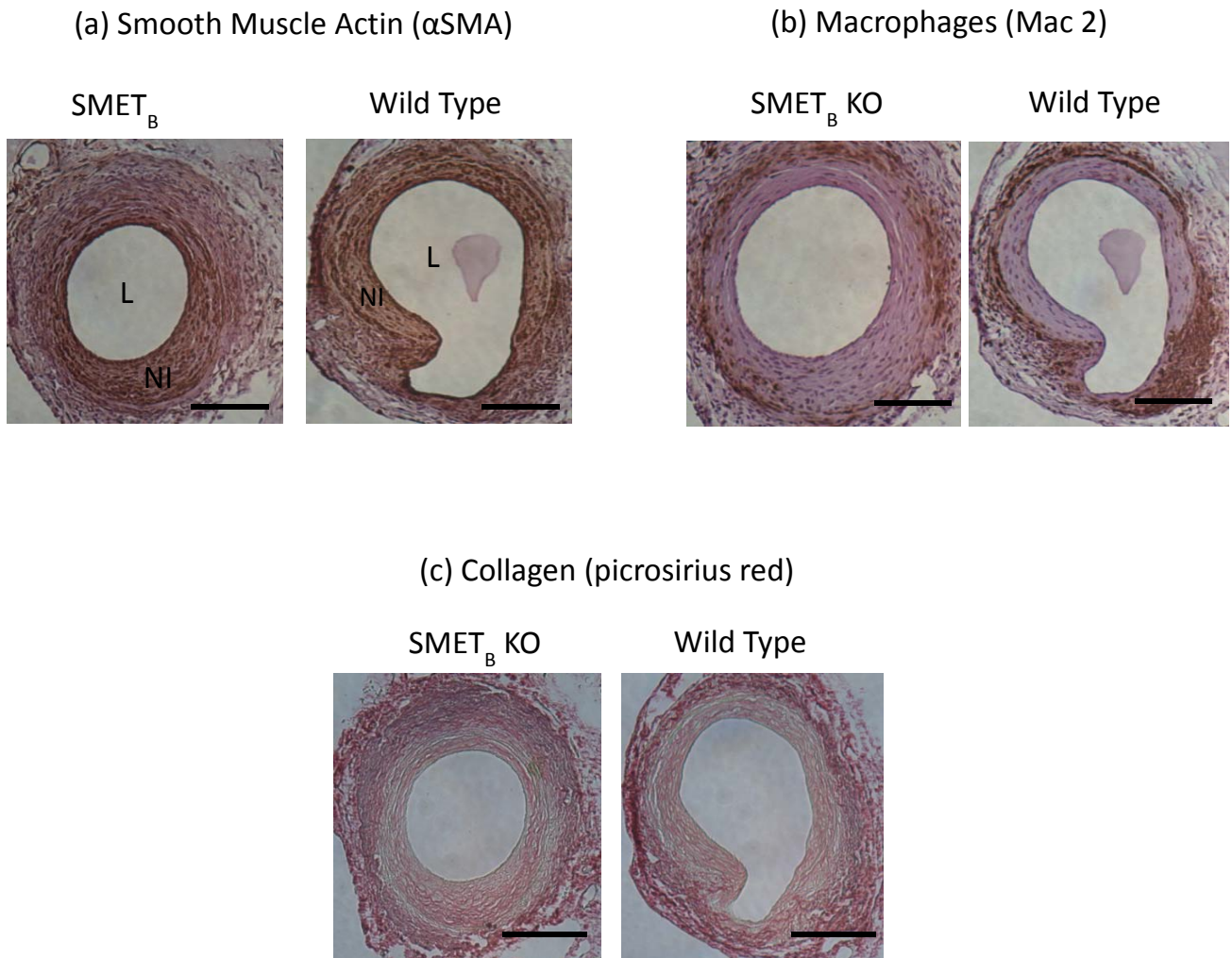
*P<0.05 compared with Vehicle. E_{max}, maximum contraction; pD₂, -log EC₅₀.

Supplementary Table S7. The impact of vascular cell selective deletion of murine vascular ET_B receptors (compared with wild type controls).

Measurement	EC ET _B KO*	SMC ET _B KO
Body Weight	NSD ^{1, 2, 3, 4}	NSD
Organ Weight	NSD ^{1, 2, 4}	NSD
Plasma [ET-1]		
Basal	Increased ²	NSD
High salt diet + ET _B antagonist		NSD
Blood Pressure (basal)		
Basal	NSD ²	Small (4mmHg) Increase
High salt diet	NSD ²	Small (4mmHg) Increase
High salt diet + ET _B antagonist.	NSD ²	NSD
Heart Rate	NSD ²	Reduced
Neointimal Proliferation	NSD ^{1, 4}	NSD
ET _B -mediated contraction		
Trachea	NSD ^{1, 2, 4}	Reduced
Mesenteric Vein	N/A	Abolished
Mesenteric Artery	N/A	Induction Abolished
Femoral Artery	N/A	N/A
ACh-mediated relaxation		
Aorta	Impaired ²	
Femoral Artery	NSD ^{1, 4}	NSD
ET-1-mediated contraction		
Femoral Artery	NSD ^{1, 4}	NSD
PE-mediated contraction		
Femoral Artery	NSD ^{1, 4}	NSD

From previous investigations. ¹Kirkby *et al.*, 2012, ²Bagnall *et al.*, 2006; ³Kelland *et al.*, 2010; ⁴Kirkby, N.S. PhD Thesis Edinburgh 2009. ACh, acetylcholine; ET-1, endothelin-1; PE, phenylephrine; ET_B, endothelin B receptor; NSD, No significant difference compared with Wild Type; N/A, not assessed.

Supplementary Figure S1

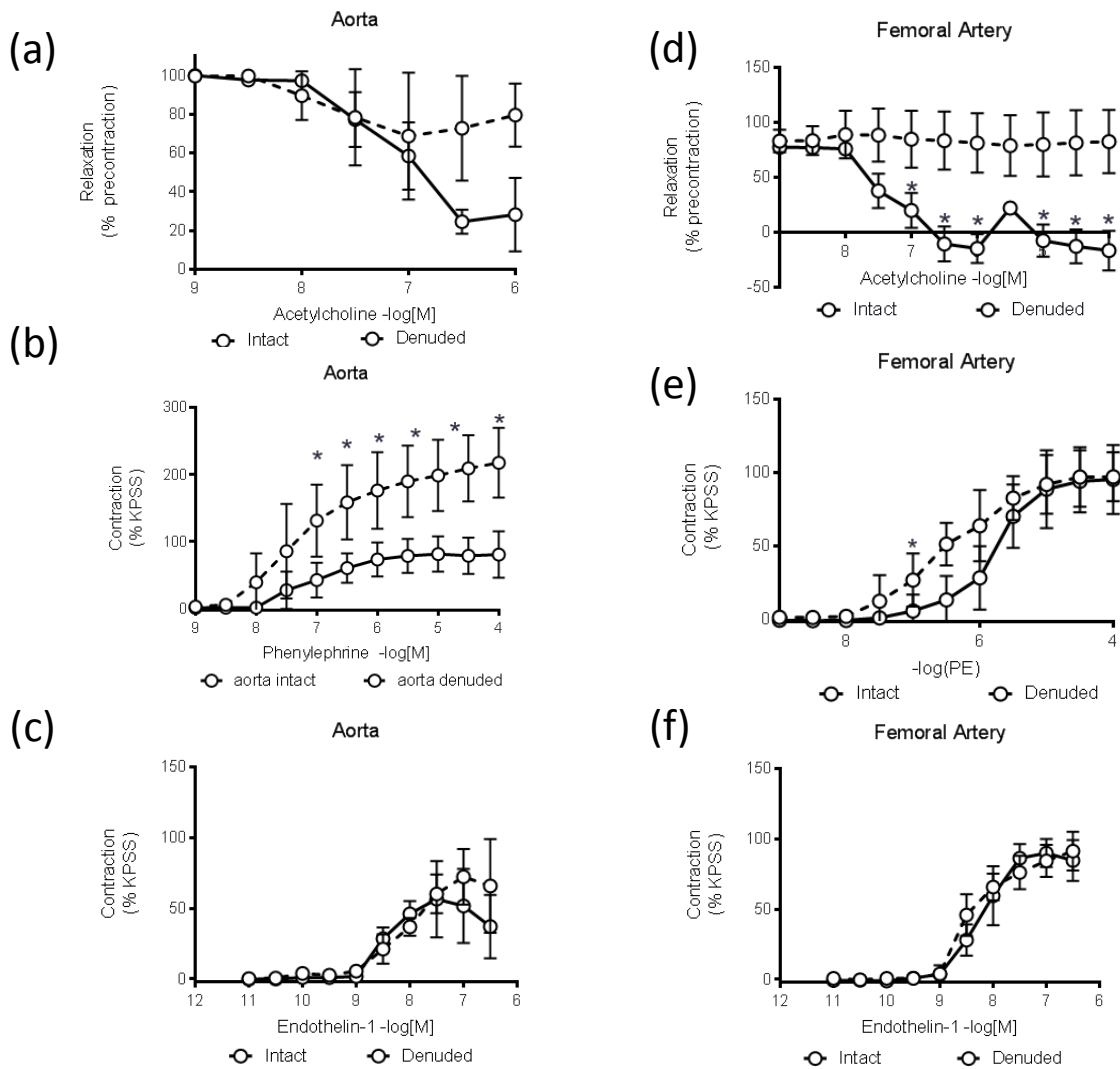


Supplementary Figure S1. Identification of smooth muscle, macrophages and collagen in neointimal lesions.

Compositional analysis of neointimal lesions induced by wire injury in smooth muscle selective ET_B KO (SMET_BKO) and wild type mouse femoral arteries showing the presence of immunoreactivity for (a) Smooth muscle actin (brown) and (b) Macrophages (Mac 2; brown). (c) Picrosirius red staining (pink) identified collagen in the neointima and media. Scale bar = 100μm. L, Lumen, NI, neointima.

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Supplementary Figure S2

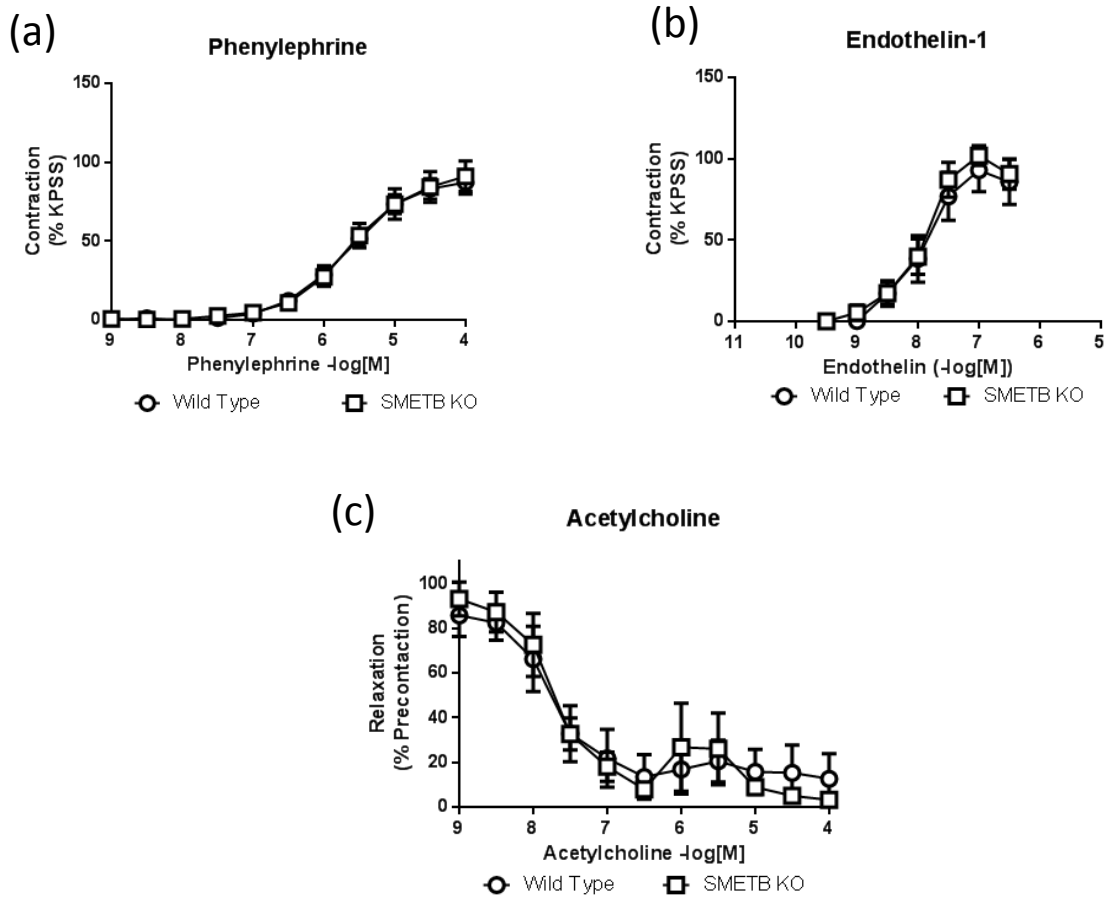


Supplementary Figure S2. Impact of endothelial cell removal on functional responses of murine aorta and femoral artery.

Aorta (a, b, c) and femoral arteries (d, e, f) from adult male C57Bl/6j mice relaxed in response to acetylcholine (ACh) (a, d) and contracted in response to phenylephrine (PE) (b, e) and endothelin-1 (ET-1) (c, f). Removal of the endothelium abolished ACh-mediated relaxation and substantially increased aortic, but not femoral arterial, contraction to PE. It did not, however, alter ET-1 mediated contraction in aorta or in femoral artery. Symbols represent mean \pm s.e.mean for n= 3 mice. *P<0.05; **P<0.01; ***P<0.001.

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Supplementary Figure S3

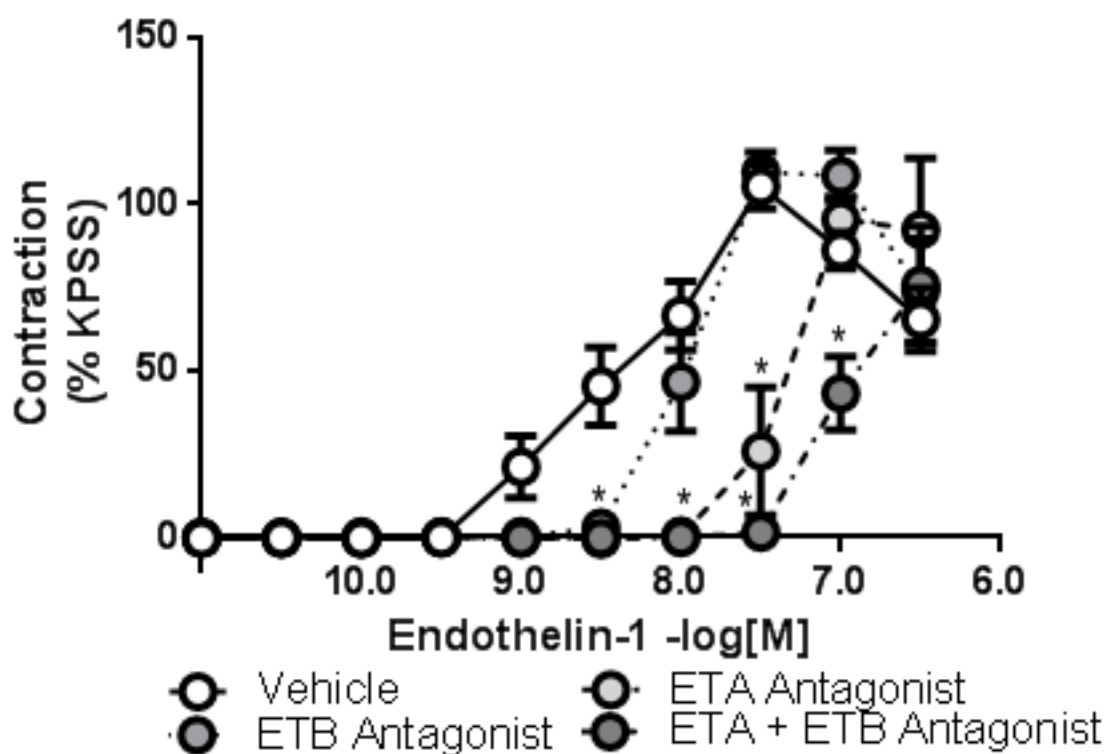


Supplementary Figure S3. Selective deletion of ET_B from the smooth muscle does not alter femoral artery function.

Concentration-response curves for (a) phenylephrine (PE), (b) endothelin-1 (ET-1) and (c) acetylcholine (ACh) were generated in femoral arteries from control (FF $^{-/-}$) and SM ET_B knockout (FFSm22Cre) mice. Responses to ACh were obtained following sub-maximal contraction with PE. Deletion of ET_B had no effect on the responses produced by these agonists. Symbols represent mean \pm s.e. mean for n=6-7 mice.

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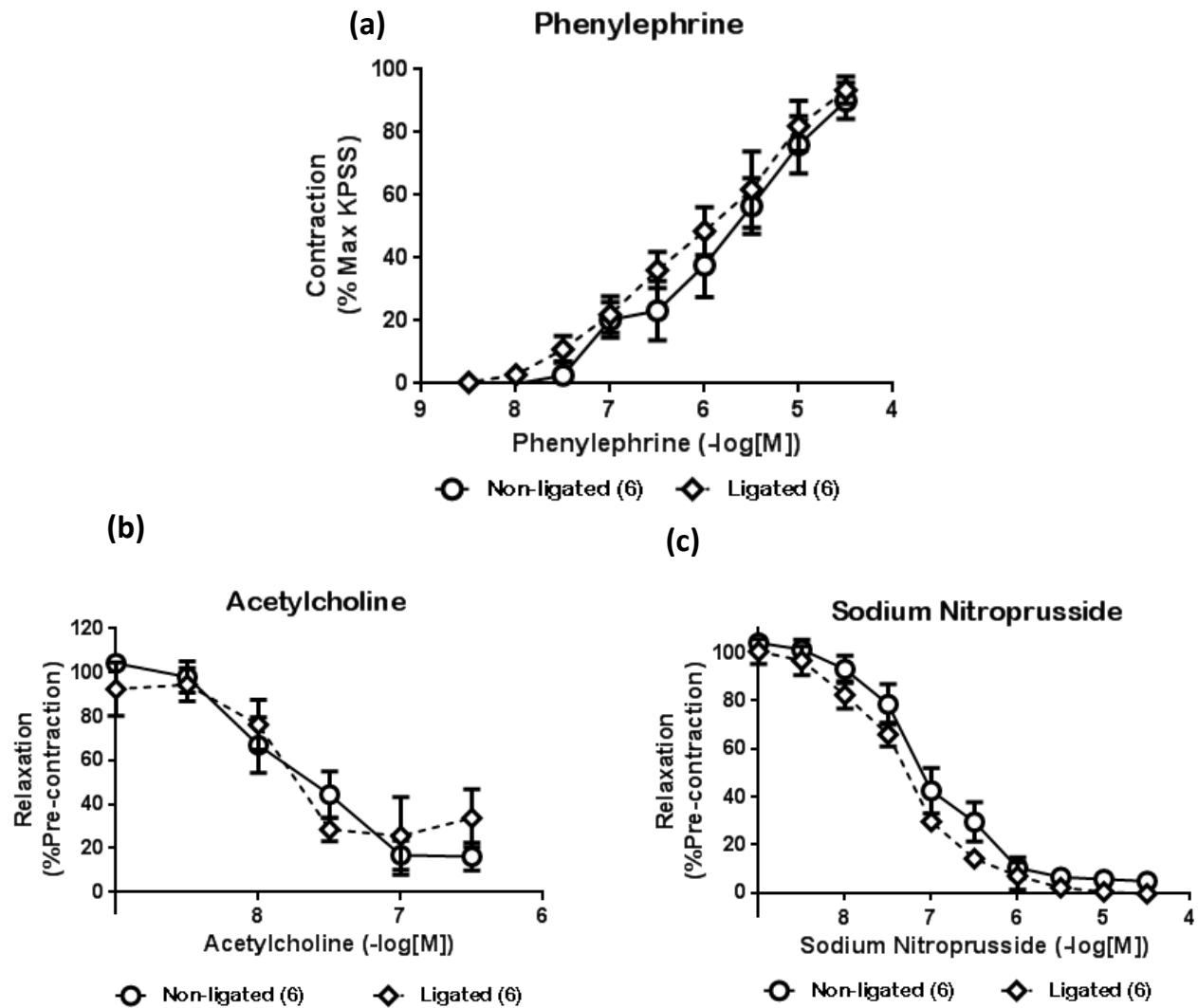
Supplementary Figure S4



Supplementary Figure S4. Endothelin-1-mediated contraction of murine mesenteric arteries is mediated by ET_A receptors.

In murine mesenteric arteries endothelin-1 (ET-1)-mediated contraction was shifted dramatically to the right by incubation with selective ET_A (BQ123; 100nM) or mixed $ET_{A/B}$ antagonism, but selective ET_B antagonism (A-192621; 100nM) had a much smaller effect. Symbols represent mean \pm s.e.mean, n=3-7. *P<0.05 compared with Vehicle.

Supplementary Figure S5



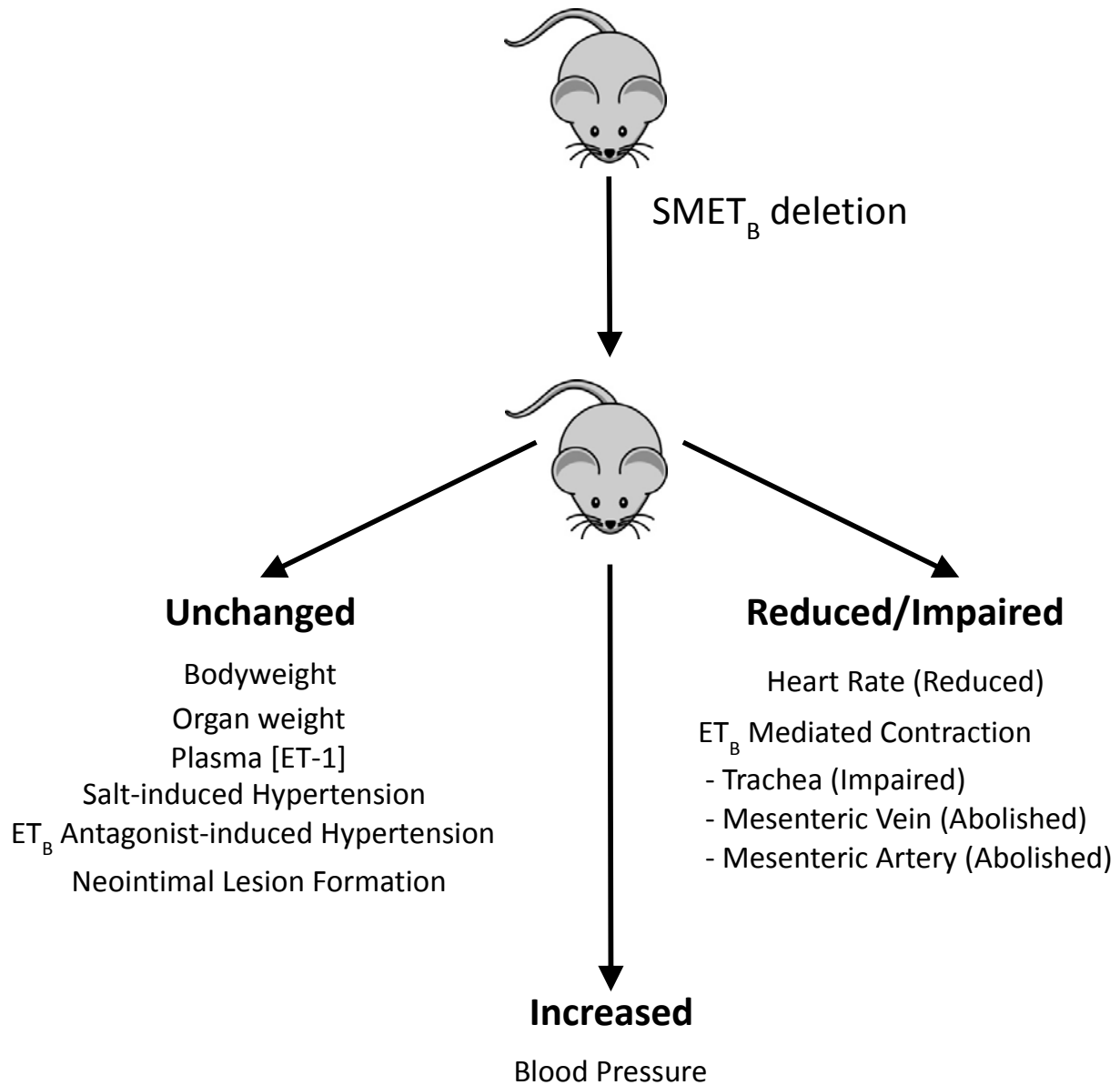
Supplementary Figure S5. Neointimal lesion formation does not alter functional responses of mouse femoral artery.

Mouse femoral arteries isolated 28 days after ligation showed unaltered responses to (a) phenylephrine (PE), (b) acetylcholine (ACh) or, (c) sodium nitroprusside (SNP)

Data are mean±s.e.mean, n=3-6.

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Supplementary Figure S6



Supplementary Figure S6. Schematic summary of the effects of smooth muscle cell specific deletion of the Endothelin B receptor (SM ET_B KO) in mice.

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